

Application No. 10/658,111
Response dated March 18, 2005
Reply to Office Action of September 30, 2004

Exhibit 1

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The role of p38 mitogen-activated protein kinase in IL-6 and IL-8 production from the TNF- α - or IL-1 β -stimulated rheumatoid synovial fibroblasts

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Received 1 October 1999; received in revised form 2 December 1999

Edited by Shozo Yamamoto

Abstract We examined the role of p38 mitogen-activated protein (MAP) kinase in the tumor necrosis factor α (TNF- α)- or interleukin-1 β (IL-1 β)-induced production of interleukin-6 (IL-6) and interleukin-8 (IL-8) in fresh rheumatoid synovial fibroblast (RSF) cultures concomitantly with the induction of p38 MAP kinase activity. Pretreatment of RSF with a specific p38 MAP kinase inhibitor, SB203580, blocked the induction of IL-6 and IL-8 without affecting nuclear translocation of nuclear factor κ B (NF- κ B) or IL-6 and IL-8 mRNA levels. These findings suggest that p38 MAP kinase inhibitor may have synergistic, rather than additive, effect for the treatment of rheumatoid arthritis.

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Key words: Rheumatoid arthritis; p38 MAP kinase; Inflammatory cytokine; NF- κ B

1. Introduction

Rheumatoid arthritis (RA) is characterized as chronic and progressive inflammatory processes of the affected joints with systemic immunological abnormalities leading to synovial hyperplasia and joint destruction. Cytokines that are abundantly produced in the inflamed rheumatoid synovial fluid, such as tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and interleukin-8 (IL-8) play crucial roles in the pathophysiology of RA. Among these cytokines, TNF- α and IL-1 β are considered indispensable for RA pathogenesis since they are known to induce IL-6, IL-8, and themselves through activation of a cellular transcription factor nuclear factor κ B (NF- κ B) [1]. On the other hand, TNF- α and IL-1 β induced a rapid increase in p38 mitogen-activated protein kinase (p38 MAP kinase) phosphorylation and the subsequent activation of its enzyme activity [2–4]. Thus, we determined the roles of p38 MAP kinase in IL-6 and IL-8 production

using a specific inhibitor SB203580 [5]. In this study we demonstrate that SB203580 inhibits IL-6 and IL-8 production without affecting the steady-state mRNA levels of IL-6 and IL-8 or blocking NF- κ B activation as viewed by its nuclear translocation.

2. Materials and methods

2.1. Reagents

Recombinant human TNF- α (TNF- α) and recombinant human IL-1 β (IL-1 β) were purchased from Roche Diagnostics. Anti-p38 MAP kinase polyclonal antibody and GST-ATF-2 (1–96) were from Santa Cruz Biotechnology. The specific antibodies to the phosphorylated form of p38 MAP kinase at threonine 180 and tyrosine 182 and to the phosphorylated ATF-2 (phosph-ATF-2) were from New England Biolabs. Protein A Sepharose CL-4B was purchased from Amersham Pharmacia Biotech. Rabbit polyclonal antibodies to human NF- κ B subunits, p65 and p50, were from Santa Cruz Biotechnology. The specific inhibitor for p38 MAP kinase SB203580, a pyridinyl imidazole compound, was purchased from Calbiochem and was dissolved in DMSO.

2.2. Cells

Rheumatoid synovial fibroblasts (RSF) were isolated as previously reported [6–8] from the fresh synovial tissue biopsy samples from active three RA patients at total arthro-replacement or arthroscopic synovectomy, as defined by the clinical criteria of the American Rheumatism Association [9]. Written informed consent was obtained from each patient. The data obtained using RSF are presented in this paper, since the same results were obtained qualitatively using RSF derived from RA patients. Briefly, the tissue samples were minced into small pieces and treated with 1 mg/ml collagenase/dispase (Roche Diagnostics) for 10–20 min at 37°C. The cells obtained were cultured in F-12 (Hams) (Life Technologies) supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 100 μ g/ml of streptomycin and 0.5 mM 2-mercaptoethanol. The culture medium was changed every 3–5 days and non-adherent lymphoid cells were removed. Adherent cell subcultures were maintained in the same medium and harvested by trypsinization (trypsin/EDTA, Life Technologies) every 7–10 days before they reached cellular confluency. All the experiments described here were conducted using the RSF during the fifth to the tenth passage to characterize the phenotype of adherent cells.

2.3. Western blot analysis

RSF cultures at approximately 80% confluency in 60 mm dishes in F-12 with various treatment were harvested and the total cell extract was prepared according to the method previously described [8]. Briefly, treated cells were washed twice with cold PBS and suspended in 0.2 ml ice-cold total cell extract (TOTEX) buffer (20 mM HEPES-KOH (pH 7.9), 350 mM NaCl, 1.0 mM MgCl₂, 20% glycerol, 1.0% NP-40, 0.5 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, 0.5 mM PMSF, 20 mM β -glycerophosphate, 0.1 mM Na₃VO₄, and 1.0 μ g/ml each of aprotinin, pepstatin, leupeptin) with scraping. The solubilized cell homogenate was harvested and centrifuged at 10000 \times g for

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Abbreviations: RA, rheumatoid arthritis; RSF, rheumatoid synovial fibroblasts; p38 MAP kinase, p38 mitogen-activated protein kinase; TNF- α , tumor necrosis factor α ; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; IL-8, interleukin-8; RT-PCR, reverse transcription-polymerase chain reaction; NF- κ B, nuclear factor κ B

30 min at 4°C, and the resultant supernatant was used for further analysis. Protein contents were determined with the DC Protein Assay kit (Bio-Rad). Equal amounts of protein were loaded on 10% SDS polyacrylamide gel. Proteins were separated electrophoretically and transferred to nitrocellulose membrane (Hybond-C Super; Amersham Pharmacia Biotech). The protein-blotted membranes were blocked with 5% (w/v) fat-free dry milk in phosphate-buffered saline with 0.05% Tween 20 (PBS-T) for over-night at 4°C. They were then incubated for 1 h at room temperature with anti-phospho p38 MAP kinase antibody at 1:500 dilution in PBS-T containing 1% bovine serum albumin. After washing three times for 5 min with PBS-T solution, blots were further incubated for 1 h at room temperature with donkey anti-rabbit IgG antibody coupled to horseradish peroxidase (Amersham Pharmacia Biotech) at 1:2000 dilution in 5% skim milk in PBS-T and washed three times in PBS-T before visualization. The phosphorylated p38 MAP kinase was detected by anti-phospho-specific p38 MAP kinase antibody by Western blot analysis and detected by SuperSignal Substrate Western Blotting (Pierce) for enhanced chemiluminescence.

2.4. Immune complex kinase assay for p38 MAP kinase activity

The p38 MAP kinase activity in RSF cultures was examined according to the method of Livingstone et al. [4]. Briefly, the soluble proteins in the cell lysate were immunoprecipitated with anti-p38 MAP kinase antibody for overnight at 4°C and further incubated with protein A Sepharose beads for 1 h. The beads were washed three times with 500 µl of ice-cold wash buffer (20 mM HEPES (pH 7.7), 50 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Triton X). The immune complex kinase assay was then performed using GST-ATF-2 (as a substrate) for 30 min at 30°C in 300 µl of kinase reaction buffer (5 µg/ml of GST-ATF-2, 20 mM HEPES (pH 7.6), 20 mM MgCl₂, 20 mM β-glycerophosphate, 2.0 mM DTT) containing 200 µM ATP. The reaction was terminated with Laemmli SDS-PAGE loading buffer and the proteins were boiled for 5 min. Equal amounts of protein samples were loaded on 10% SDS-PAGE. The gel was blotted on a nitrocellulose membrane (Hybond-C super). After membranes were blocked with 5% (w/v) fat-free dry milk in PBS-T for overnight at 4°C, they were incubated at room temperature for 1 h with phospho-ATF-2 antibody at 1:1000 dilution. Then, after washing for three times with PBS-T, they were reacted with the secondary antibody, donkey anti-rabbit IgG antibody coupled to horseradish peroxidase, at 1:2000 dilution. Blots were washed three times in PBS-T before visualization using SuperSignal Substrate Western Blotting.

2.5. Cytotoxicity assay

In order to examine the cytotoxicity of SB203580, the cell viability of RSF upon treatment with SB203580 at various concentrations of this compound was determined using WST-1 (Roche Diagnostics) according to the manufacturer's protocol. RSF cultures were incubated with SB203580 in a 96 well plate, incubated for 4 h in the presence of WST-1, and the dissolved formazan was measured at 450 nm by spectrophotometry.

2.6. Cytokine assays

The cytokine concentrations in RSF culture supernatant under various conditions were determined using cytokine-specific ELISA kits for IL-6, IL-8 and VEGF (Biotrak human ELISA kits; Amersham Pharmacia Biotech). All the procedures were carried out as recommended by the manufacturer. Triplicates were used for each test condition in the three independent cultures. The statistical significances of difference in the mean cytokine production were evaluated by the *t*-test.

2.7. Immunofluorescence

In order to determine subcellular localization of NF-κB, RSF were cultured in four well LabTek chamber slides (Nalge Nunc International) and allowed to adhere for 72 h. Cells were then stimulated with 10 ng/ml TNF-α or 20 ng/ml IL-1β with or without pretreatment for 1 h with 30 µM SB203580. Indirect immunofluorescence staining using specific anti-NF-κB antibodies was performed as reported [6–8]. Briefly, the cells were fixed in PBS containing 4.5% paraformaldehyde for 10 min at room temperature and then permeabilized by 0.5% Triton X-100 in PBS for 20 min at room temperature. They were then incubated with rabbit polyclonal antibody against p65 or p50 NF-κB subunits (Santa Cruz Biotech) for 45 min at 37°C. After washing with PBS, the cells were incubated with FITC-conjugated goat

anti-rabbit (whole IgG) antibody (Cappel Organon Teknika, Durham, NC, USA) for 20 min at 37°C. Subcellular localization was determined using a fluorescent microscope.

2.8. Semi-quantitation of IL-6 and IL-8 mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared from RSF with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Amplification of IL-6, IL-8, and β-actin mRNA was performed using a commercial RT-PCR system (Titan[®] One Tube RT-PCR System; Roche Diagnostics). Gene specific oligonucleotide primers to human IL-6, IL-8 and human β-actin were purchased from CLP. PCR reactions were performed according to the manufacturer's protocol and the products were analyzed following 5, 10, 15, 20, or 30 cycles of amplification and were resolved on a 1.5% agarose gel, stained by ethidium bromide and visualized under an ultraviolet (UV) light.

3. Results

3.1. Activation of p38 MAP kinase by TNF-α during induction of IL-6 and IL-8

TNF-α and IL-1β are known to induce production of various cytokines including IL-6 and IL-8 in fibroblasts [6–8]. In order to examine the involvement of p38 MAP kinase in induction of IL-6 and IL-8 by TNF-α or IL-1β, we have examined if p38 MAP kinase is activated in RSF cultures. In Fig. 1A, RSF were treated with TNF-α (10 ng/ml) and the p38 MAP kinase activity was measured by immune complex kinase assay using recombinant GST-ATF2 protein as a substrate. The increase of p38 MAP kinase activity was observed

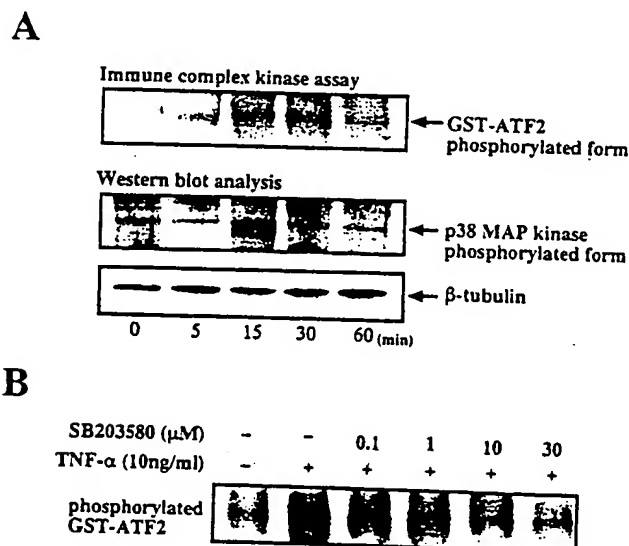


Fig. 1. Activation of p38 MAP kinase in the fresh RSF cultures and the effect of a p38 MAP kinase inhibitor SB203580. A: In vitro activation of p38 MAP kinase. RSF were treated with 10 ng/ml of TNF-α and p38 MAP kinase activity was measured by immune complex kinase assay using recombinant GST-ATF2 protein as a substrate (upper panel). The phosphorylated form of p38 MAP kinase at Thr-180 and Tyr-182 was demonstrated by Western blot analysis using a specific antibody against the phosphorylated form of p38 MAP kinase (lower panel). The same protein samples were probed with antibody to β-tubulin as an internal control. B: Effects of SB203580 on p38 MAP kinase activity detected by immune complex kinase assay. RSF were pretreated for 1 h with various concentrations (0.1, 1, 10, 30 µM) of SB203580 and were stimulated with 10 ng/ml of TNF-α for 30 min. The cell lysate was immunoprecipitated with anti-p38 MAP kinase antibody and the p38 MAP kinase activity was measured with purified recombinant GST-ATF2 as described in Section 2.

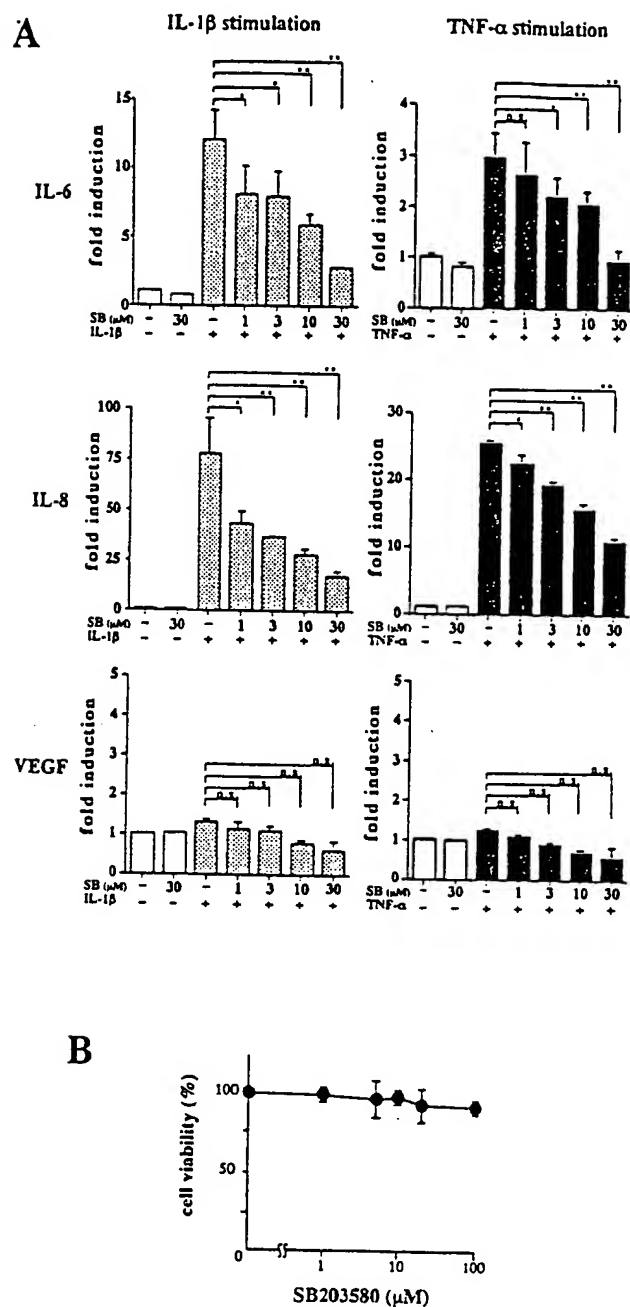


Fig. 2. Effects of a SB203580 on the induced production of IL-6 and IL-8 in RSF. The levels of IL-6 and IL-8 were measured by ELISA systems. At least three independent RSF cultures were examined and the representative results are shown. The experiments were performed in triplicates and the results represent the mean \pm S.D. A: SB203580 (SB) inhibited IL-6 and IL-8 production in a dose-dependent manner. Cells were treated with SB203580 for 1 h prior to stimulation with IL-1 β (20 ng/ml) (\square) or TNF- α (10 ng/ml) (\blacksquare) for 12 h. (\square) means no treatment with TNF- α or IL-1 β . Statistical significance: *, $P < 0.05$; **, $P < 0.01$; n.s., not significant. B: Cytotoxicity of SB203580 in RSF cultures.

as fast as 15 min of stimulation and reached its maximum after 30 min by TNF- α treatment (about six fold increase by densitometric measurement). Moreover, we demonstrated, in the lower panel of Fig. 1A, that the amount of phosphorylation form of p38 MAP kinase at Thr-180 and Tyr-182 was readily increased as demonstrated by Western blot analysis using a specific antibody against the phosphorylated form of

p38 MAP kinase (at Thr-180 and Tyr-182). These phosphorylations on p38 MAP kinase, indicating its activation, became detectable after 15 min of TNF- α stimulation and disappeared after 60 min, which was consistent with the results of immune complex kinase assay (upper panel). Similar results were obtained when RSF were stimulated with IL-1 β (data not shown).

These results establish that the p38 MAP kinase pathway is rapidly activated in RSF by the actions of proinflammatory cytokine as previously reported with various cell lines [3,10].

In Fig. 1B, RSF were pretreated for 1 h with SB203580 and stimulated with 10 ng/ml of TNF- α for 30 min. Cell lysates were prepared and subjected to the immune complex kinase assay using GST-ATF2 as a substrate. The TNF- α treatment stimulated p38 MAP kinase activity. However, pretreatment with SB203580 prevented this increase of p38 MAP kinase activity in a dose-dependent manner. At 30 μ M SB203580, p38 MAP kinase activity was inhibited almost to the basal level.

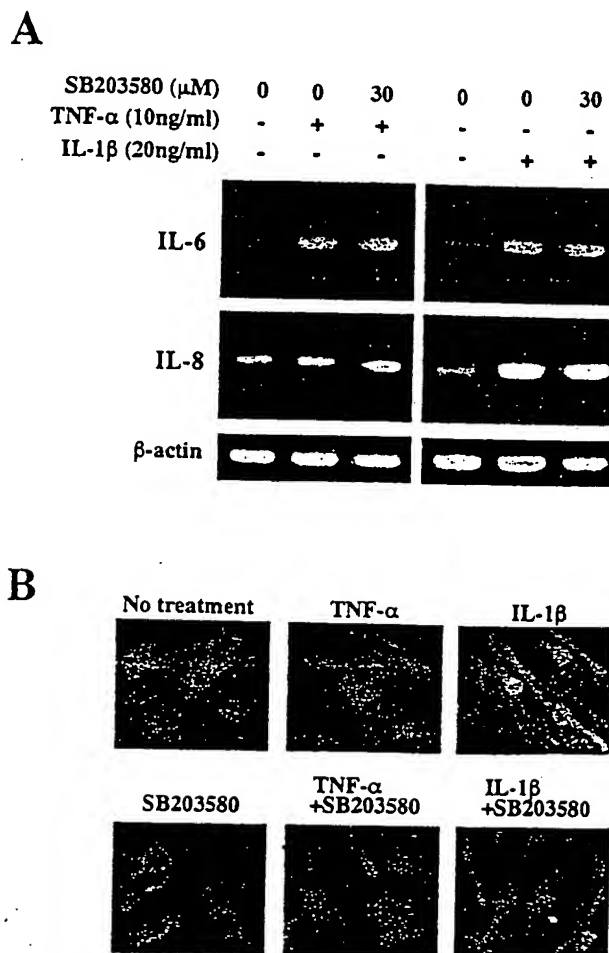


Fig. 3. Transcription of IL-6 or IL-8 was not affected by the p38 MAP kinase inhibitor SB203580. A: The effect of SB203580 on the steady-state level of IL-6 and IL-8 mRNA. RSF were pretreated for 1 h with 30 μ M of SB203580 and stimulated for 12 h with 10 ng/ml of TNF- α (left) or 20 ng/ml of IL-1 β (right). The steady-state mRNA levels for IL-6 and IL-8 were examined by RT-PCR. B: The effect of SB203580 on the nuclear translocation of NF- κ B that was induced by TNF- α or IL-1 β . Indirect immunofluorescence was carried out with rabbit polyclonal antibody against NF- κ B subunit p65. The cells were similarly treated with SB203580 and TNF- α or IL-1 β as in (A).

3.2. Suppression of the IL-6 and IL-8 induction by a p38 MAP kinase inhibitor SB203580

In order to evaluate the effect of p38 MAP kinase on the induction of IL-6/IL-8 production by TNF- α or IL-1 β , the effect of SB203580, a p38 MAP kinase inhibitor, was examined. As shown in Fig. 2, RSF cultures were stimulated with TNF- α (10 ng/ml) or IL-1 β (20 ng/ml), and concentrations of IL-6 and IL-8 in the cell culture supernatant were measured after 12 h of stimulation. After 12 h of stimulation by TNF- α (10 ng/ml), the extents of augmentation were 3 and 26 fold for IL-6 and IL-8, respectively. Similarly, after 12 h of stimulation by IL-1 β (20 ng/ml), IL-6 and IL-8 production were augmented by 12 and 75 fold, respectively. When the RSF was pretreated with various concentrations of SB203580 (up to 30 μ M) for 1 h before the treatment with TNF- α or IL-1 β , the extents of induction of IL-6 and IL-8 by either TNF- α or IL-1 β were significantly reduced by SB203580 in a dose-dependent manner. Similar profiles of suppression were observed with RSF cultures from other RA patients (data not shown). The basal levels of IL-6 and IL-8 production, without TNF- α or IL-1 β treatment, were not significantly affected. For example, at 10 μ M SB203580 the IL-1 β -stimulated IL-6 and IL-8 production were inhibited by 50.8% and 53%, respectively (both statistically significant, $P < 0.01$). Similarly, the TNF- α -mediated inductions were inhibited by 30.7% and 38.5% for IL-6 and IL-8, respectively ($P < 0.01$). In contrast, the concentration of VEGF, known not to be under the control of NF- κ B, in the supernatant of RSF cultures stimulated by TNF- α or IL-1 β were not significantly reduced. Moreover, SB203580 at these concentrations did not show significant cytotoxicity (Fig. 2B). These findings indicate that a p38 MAP kinase inhibitor SB203580 suppressed the levels of IL-6 and IL-8 that were induced by TNF- α or IL-1 β at non-cytotoxic concentrations. Thus, the effect of p38 MAP kinase on IL-6 and IL-8 production appeared to be specific.

3.3. The lack of evidence that p38 MAP kinase inhibitor acts on IL-6 and IL-8 transcription

Since it was reported previously that p38 MAP kinase is involved in gene expression at the post-transcriptional level [11,12], we examined the effect of SB203580 on the steady-state level of IL-6 and IL-8 mRNA. RSF was pretreated for 1 h with 30 μ M of SB203580, and then TNF- α (10 ng/ml) or IL-1 β (20 ng/ml) was added for a further 12 h. Total RNA samples were prepared from these cells and the steady-state mRNA levels for IL-6 and IL-8 were examined by RT-PCR. A series of PCR reactions were monitored for amplification of IL-6 and IL-8 mRNA at 5, 10, 15, 20 and 30 cycles of PCR. At PCR cycles over 20, there was no difference in intensity of the gene-specific bands. Thus, the results shown in Fig. 3A represent the amplified band for IL-6 and IL-8 mRNA detected at 15 cycles of the RT-PCR. As demonstrated in Fig. 3A, SB203580 even at the highest concentration (30 μ M) did not significantly inhibit the induction of IL-6 and IL-8 mRNA. Since NF- κ B is known to play a crucial role in IL-6 and IL-8 induction as a positive transcriptional regulator, we examined whether SB203580 could affect the nuclear translocation of NF- κ B, as an indication of its signal-induced activation, stimulated by TNF- α or IL-1 β . To determine the subcellular distribution of NF- κ B, indirect immunofluorescence was performed with rabbit polyclonal antibody against NF- κ B subunit p65. As shown in Fig. 3B, p65 was localized in

the cytoplasm of unstimulated RSF. When the cells were stimulated with 10 ng/ml of TNF- α or 20 ng/ml of IL-1 β , p65 was translocated to the nucleus within 30 min as we previously reported [6–8,13]. We further examined whether this nuclear translocation could be blocked by SB203580. When RSF were pretreated for 1 h with SB203580 (30 μ M), at which concentration the induction of IL-6 and IL-8 production was remarkably suppressed (Fig. 3A), the nuclear translocation of NF- κ B was not blocked.

4. Discussion

Accumulating evidences have incriminated various cytokines and their interactions in pathophysiology of RA [8]. Among these cytokines, TNF- α and IL-1 β are considered to play crucial roles [6–8]. These notions have been confirmed by demonstration of clinical efficacies of antibodies against TNF- α [14,15], IL-6 [16] and IL-1 antagonists [17] in the treatment of RA synovitis. Induction of IL-6 and IL-8 by IL-1 β or TNF- α is mediated by intracellular signal transduction cascades involving a number of protein kinases. In addition to a common kinase pathway involving I κ B kinases that lead to NF- κ B activation [1,18,19], both IL-1 β and TNF- α signaling cascades include three distinct types of MAP kinases (p42/p44, p54/JNK, and p38) [10,20].

We were particularly interested in p38 MAP kinase since SB203580 had been initially identified as a potent inhibitor of inflammatory cytokine production from THP-1 cells by random screening and p38 MAP kinase was identified as the specific target molecule [5]. In fact, previous reports have demonstrated that p38 MAP kinase is involved in the induction of inflammatory cytokines [5,10,21,22].

In this paper we confirmed that in the fresh RSF cultures prepared from RA patients either TNF- α or IL-1 β could induce phosphorylation (and activation) of p38 MAP kinase within 15 min followed by augmented production of IL-6 and IL-8 (Fig. 1A). As expected, SB203580 could block p38 MAP kinase activity and cytokines (IL-6 and IL-8) induction in RSF (Figs. 1B and 2). Although earlier studies reported that p38 MAP kinase appeared to be involved in the nuclear translocation (signal-induced activation) of NF- κ B and transcriptional initiation of the genes under the control of NF- κ B [23,24], we did not see any effect of SB203580 on the NF- κ B nuclear translocation nor steady-state levels of IL-6 and IL-8 mRNA even at the highest concentration of SB203580 (Fig. 3), which was consistent with recent studies by others using fibroblast cell lines.

There are a number of reports regarding the mechanism of SB203580 in blocking the induced production of inflammatory cytokines such as IL-6 and IL-8 by stimulation with proinflammatory cytokines, TNF- α or IL-1 β [6,25]. However, the mechanism of its action is still controversial. For example, Beyaert et al. [3] reported that the NF- κ B-dependent gene expression was inhibited by SB203580 in a mouse fibrosarcoma cell line L929 stably transfected with a NF- κ B-dependent chloramphenicol acetyl transferase (CAT) plasmid. Yet, SB203580 did not affect the TNF-induced DNA-binding of NF- κ B or phosphorylation of NF- κ B or I κ B. In contrast, Miyazawa et al. [26] showed that SB203580 did not block transient expression of luciferase gene under the control of IL-6 promoter in a transient assay with synovial fibroblasts. They claimed that the effect of SB203580 might be post-trans-

scriptional since the transcriptional initiation of the TNF-induced IL-6 mRNA level was not suppressed at all by SB203580 in a nuclear run-on assay using the isolated nuclei of the TNF-stimulated synovial fibroblasts. Similarly to our observations, Caivano [11] and Pietersma et al. [12] reported that SB203580 blocked production of inducible nitric oxide synthetase and intercellular cell adhesion molecule 1, respectively, without affecting the mRNA levels as observed by RT-PCR.

Our findings together with others demonstrated clearly that p38 MAP kinase is involved in the TNF- α - or IL-1 β -induced production of inflammatory cytokines independently from the NF- κ B cascade. Many of anti-rheumatic drugs currently used for the treatment of RA patients are known to block the NF- κ B pathway at various steps, such as inhibition of I κ B kinases by aspirin [27,28] and Sulindac [29] and inhibition of NF- κ B DNA-binding by gold [7,30]. Thus, use of p38 MAP kinase inhibitors such as SB203580 and its derivatives should have synergistic, rather than additive, effects in combination with conventional anti-rheumatic drugs in blocking the production of inflammatory cytokines, cell adhesion molecules and inducible nitric oxide synthetase that participate in the maintenance and expansion of rheumatoid inflammation. It may also circumvent side effects of conventional anti-rheumatic therapy without losing therapeutic efficacies.

Acknowledgements: This work was supported by grants-in-aid from the Ministry of Health and Welfare, the Ministry of Education, Science and Culture of Japan, Japan Human Science Foundation, Japan Rheumatism Foundation and Aichi D.R.G. Foundation.

References

- [1] Mukaida, N., Mahe, Y. and Matsushima, K. (1990) *J. Biol. Chem.* 265, 21128–21133.
- [2] Wesselborg, S., Bauer, M.K.A., Vogt, M., Schmitz, M.L. and Schulze-Osthoff, K. (1997) *J. Biol. Chem.* 272, 12422–12429.
- [3] Beyaert, R., Cuenda, A., Berghe, W.V., Plaisance, S., Lee, J.C., Haegeman, G., Cohen, P. and Fiers, W. (1996) *EMBO J.* 15, 1914–1923.
- [4] Livingstone, C., Patel, G. and Jones, N. (1995) *EMBO J.* 14, 1785–1797.
- [5] Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthal, M.J., Heys, J.R., Landvatter, S.W., Stricker, J.E., McLaughlin, M.M., Siemens, I.R., Fisher, S.M., Livi, G.P., White, J.R., Adams, J.L. and Young, P.R. (1994) *Nature* 372, 739–746.
- [6] Sakurada, S., Kato, T. and Okamoto, T. (1996) *Int. Immunol.* 8, 1483–1493.
- [7] Yoshida, S., Kato, T., Sakurada, S., Kurono, C., Yang, J.P., Matsui, N., Soji, T. and Okamoto, T. (1999) *Int. Immunol.* 11, 151–158.
- [8] Yoshida, S., Katoh, T., Tetsuka, T., Uno, K., Matsui, N. and Okamoto, T. (1999) *J. Immunol.* 163, 351–358.
- [9] Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S., Medsger Jr., M.A., Michell, D.M., Neustadt, D.H., Pinals, R.S., Schaller, J.G., Sharp, J.T., Wilder, R.L. and Hunder, G.G. (1987) *Arthritis Rheum.* 31, 315–324.
- [10] Ringeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J. and Davis, R.J. (1995) *J. Biol. Chem.* 270, 7420–7426.
- [11] Caivano, M. (1998) *FEBS Lett.* 429, 249–253.
- [12] Pietersma, A., Tilly, B.C., Gaestel, M., de Jong, N., Lee, J.C., Koster, J.F. and Sluiter, W. (1997) *Biochem. Biophys. Res. Commun.* 230, 44–48.
- [13] Hayashi, T., Sekine, T. and Okamoto, T. (1993) *J. Biol. Chem.* 268, 26790–26795.
- [14] Brennan, F.M., Chantry, D., Jackson, A., Maini, R. and Feldmann, M. (1989) *Lancet* 29, 244–247.
- [15] Elliott, M.J., Maini, R.N., Feldmann, M., Long-Fox, A., Charles, P., Katsikis, P., Brennan, F.M., Walker, J., Bijl, H., Ghayeb, J. and Woody, J.N. (1993) *Arthritis Rheum.* 36, 1681–1690.
- [16] Wendling, D., Racadot, E. and Wijdenes, J. (1993) *J. Rheumatol.* 20, 259–262.
- [17] Bendele, A., McAb Sennello, G., Frazier, J., Chlipala, E. and McCabe, D. et al. (1999) *Arthritis Rheum.* 42, 498–506.
- [18] Woronicz, J.D., Gao, X., Cao, Z., Rothe, M. and Goeddel, D.V. (1997) *Science* 278, 866–869.
- [19] Okamoto, T., Sakurada, S., Yang, J.P. and Merin, J.P. (1997) *Curr. Top. Cell. Regul.* 35, 149–161.
- [20] Cobb, M.H. and Goldsmith, E.J. (1995) *J. Biol. Chem.* 270, 14843–14846.
- [21] Eliopoulos, A.G., Gallager, N.J., Blake, S.M.S., Dawson, C.W. and Young, L.S. (1999) *J. Biol. Chem.* 274, 16085–16096.
- [22] Krause, A., Holtmann, H., Eickemeier, S., Winzen, R., Szamel, M., Resch, K., Saklatvala, J. and Kracht, M. (1998) *J. Biol. Chem.* 273, 23681–23689.
- [23] Baldassare, J.J., Bi, Y. and Bellone, C.J. (1999) *J. Immunol.* 162, 5367–5373.
- [24] Vanden, B.W., Plaisance, S., Boone, E., De Bosscher, K., Schmitz, M.L., Fiers, W. and Haegeman, G. (1998) *J. Biol. Chem.* 273, 3285–3290.
- [25] Zhang, Y.H. and Lin, J.X. (1990) *J. Mol. Cell Biol.* 10, 3818–3823.
- [26] Miyazawa, K., Mori, A., Miyata, H., Akahane, M., Ajiwaka, Y. and Okudaira, H. (1998) *Arthritis Rheum.* 41, 2483–2488.
- [27] Kopp, E. and Ghosh, S. (1994) *Science* 265, 956–959.
- [28] Weber, C., Erl, W., Pietsch, A. and Weber, P.C. (1995) *Circulation* 91, 1914–1917.
- [29] Yamamoto, Y., Yin, M.J., Lin, K.M. and Gaynor, R.B. (1998) *J. Biol. Chem.* 273, 27307–27314.
- [30] Yang, J.P., Meirin, J.P., Nakano, T., Kato, T., Kitabe, Y. and Okamoto, T. (1995) *FEBS Lett.* 361, 89–96.

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- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
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- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
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